PRODUCTION OF LACTIC ACID FROM BANANA STEM WASTE USING MIXED CULTURE

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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JUNE 2014

ABSTRACT

Lactic acid is a chemical compound that plays a role in various biochemical processes and also widely used in the food, cosmetic, pharmaceutical, and chemical industries. Lactic acid can be obtained by either microbial fermentation or chemical synthesis. Presently more than 95 % of industrial production of lactic acid is based on fermentation by using lactic acid bacteria. The research was done to produce lactic acid by using mixed culture of facultative anaerobic bacteria and banana stem waste. By using BSW as substrate and PMD as inoculums, the process was done according to selected factors. Source of inoculums (A), agitation (C) and ratio of substrate to inoculums (D) are categorical factors while temperature (B) and fermentation time (E) are numerical factors. Two factor A are used, Yakult and Nutrigen; three levels of factor B, 30, 35, 40 ^{o}C; agitate or non-agitate for factor C; while factor D used, 4:1 and 2:3; and also three levels of factor E, 20, 30, 40 hours. Design Expert 6.0 software is used to set the total number of experimental run, suggested validation experiment and also the best condition to produce lactic acid. The samples were analyzed using High Performance Liquid Chromatography while glucose content in the BSW is determined using Dinitrosalicylic Colorimetric (DNS) method and Ultra Violet-Visible (UV-Vis) Spectrometer. Factor A contributes the most in lactic acid fermentation with percentage of 1.07%. During preliminary experiments, Yakult and Nutrigen have respectively initial pH of 3.74 and 3.77 and decreases to 3.36 and 3.58 at the end of experiment. Previous study stated that when pH of the culture decreases, the concentration of lactic acid increases. Thus, it is showed that factor A could contribute the most in producing lactic acid. Factor's interaction between factor C and E contributes the most which is 35.55%. A total of five validation runs were suggested in order to confirm the experiment result. All runs have error between experimental and predicted lactic acid yield for about 8 – 35%. The best conditions to produce lactic acid for this research were at temperature 39^{o}C, no agitation, 20 hours of fermentation time and only 8% error with predicted lactic acid yield of 0.1009 g/g and experimental lactic acid yield of 0.0925 g/g. The production of lactic acid is affected by glucose content in the substrate used, temperature, agitation and fermentation time. Utilization of total sugar increased as the fermentation time increased, reducing the available sugar content in the media, thus increasing lactic acid production. Therefore, the result obtained in the study showed that BSW can be used as substrate in producing lactic acid and the parameter tested does affect the production of lactic acid.

ABSTRAK

Asid laktik adalah sebatian kimia yang memainkan peranan dalam pelbagai proses biokimia dan juga digunakan secara meluas dalam industri makanan , kosmetik , farmaseutikal, dan kimia. Asid laktik boleh didapati sama ada dengan penapaian mikrob atau sintesis kimia. Pada masa ini lebih daripada 95 % daripada pengeluaran perindustrian asid laktik adalah berdasarkan kepada penapaian dengan menggunakan bakteria asid laktik. Kajian ini dilakukan untuk menghasilkan asid laktik dengan menggunakan kultur campuran bakteria anaerobik fakultatif dan sisa batang pisang . Dengan menggunakan BSW sebagai substrat dan PMD sebagai inoculums , proses itu dilakukan mengikut faktor dipilih. Sumber inoculums (A) , putaran (C) dan nisbah substrat ke inoculums (D) adalah faktor mutlak manakala suhu (B) dan masa penapaian (E) adalah faktor berangka. Dua faktor A digunakan, Yakult dan Nutrigen ; tiga tahap faktor B, 30, 35 , 40 ^{o}C ; putaran atau tiada putaran untuk faktor C; sementara faktor D digunakan, 4:1 dan 2:3 ; dan juga tiga paras faktor E, 20, 30, 40 jam. Perisian 'Design Expert 6.0' digunakan untuk menetapkan jumlah jangka eksperimen , mencadangkan eksperimen pengesahan dan juga keadaan terbaik untuk menghasilkan asid laktik. Sampel dianalisis dengan menggunakan 'High Performance Liquid Chromatography' manakala kandungan glukosa dalam BSW ditentukan menggunakan kaedah 'Dinitrosalicylic Colorimetric' (DNS) dan 'Ultra Violet – Visible (UV -Vis) Spectrometer' . Faktor A menyumbang yang paling tinggi dalam penapaian asid laktik dengan peratusan sebanyak 1.07% . Semasa eksperimen awal, Yakult dan Nutrigen mempunyai pH masing-masing awal 3.74 dan 3.77 dan berkurangan kepada 3.36 dan 3.58 pada akhir eksperimen. Kajian terdahulu menunjukkan bahawa apabila pH kultur berkurangan, kepekatan asid laktik meningkat. Oleh itu, ia menunjukkan faktor yang A boleh menyumbang paling tinggi dalam menghasilkan asid laktik. Interaksi antara faktor C dan E menyumbang sebanyak 35.55 %. Sebanyak lima pengesahan eksperimen telah dicadangkan untuk mengesahkan hasil percubaan. Semua eksperimen mempunyai ralat di antara hasil asid laktik eksperimen dan diramalkan untuk kira-kira 8 - 35% . Faktor-faktor yang terbaik untuk menghasilkan asid laktik untuk kajian ini berada pada 39^{o}C suhu , tiada putaran, 20 jam masa penapaian dan ralat hanya 8 % dengan meramalkan hasil asid laktik daripada 0.1009 g / g dan eksperimen hasil asid laktik daripada 0. 0925 g / g. Pengeluaran asid laktik dipengaruhi oleh kandungan glukosa dalam substrat yang digunakan, suhu , putaran dan penapaian masa. Penggunaan daripada jumlah gula meningkat sebagai masa penapaian meningkat , mengurangkan kandungan gula yang terdapat di media, sekali gus meningkatkan pengeluaran asid laktik. Oleh itu, keputusan yang diperolehi dalam kajian ini menunjukkan bahawa BSW boleh digunakan sebagai substrat dalam menghasilkan asid laktik, dan faktor yang diuji boleh menjejaskan pengeluaran asid laktik.

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LIST OF ABBREVIATIONS

^{o}C	Degree Celcius
rpm	Revolution per minute
%	Percentage

ANOVA	Analysis of variance
BSW	Banana stem waste
C	Concentration
DNS	Dinitrosalicylic Colorimetric
FFD	Fractional Factorial Design
HPLC	High Performance Liquid Chromatography
PMD	Probiotic milk drinks
S:I	Substrate to inoculums
SSF	Solid state fermentation
UV-Vis	Ultra Violet-Visible
Y	Yield

1 INTRODUCTION

1.1 Motivation and statement of problem

Lactic acid is a chemical compound that plays a role in various biochemical processes enough to grabs all the attention as a chemical with many potential applications. Lactic acid is widely used in the food, cosmetic, pharmaceutical, and chemical industries and has received widely interest for use as a monomer for the biodegradable poly (lactic acid) production (Mudaliyar *et al*., 2011). Besides, lactic acid is also used commercially in the meat processes industry, together with hams, fish and poultry. It's also provide products with an increase in shelf life, better controls of food borne pathogens and enhanced flavour (Nagarjun *et al*., 2004). Presently the main growing application of lactic acid is in the production of biodegradable and renewable raw material based poly lactic acid (PLA) polymers (Sheeladevi *et al*., 2011)

Lactic acid can be obtained by either microbial fermentation or chemical synthesis and presently more than 95 % of industrial production of lactic acid is based on fermentation (Wee *et al*., 2006). Fermentation is known to be happened in anaerobic condition. Through fermentation, lactic acid bacteria also have the ability to produce lactic acid from sugar.

Lactic acid bacteria are generally considered facultative anaerobic obligate fermentative bacteria (Brooijmans, 2008). Facultative anaerobic bacteria are microorganisms which grow in the presence or absence of air (Condon, 1983). Holzapfel *et al.,* (1995) stated that lactic acid bacteria produce various metabolic products with antimicrobial properties such as organic acids like lactic acid and acetic acid. Lactic acid bacteria (LAB) are the most commonly used as probiotics. Probiotics are a live microbial food and feed supplement, which beneficially affects host by improving the intestinal microbial balance (Thongheam *et al*., 2008). According to Elmarzugi *et al*., (2010), most of commercially used probiotic products include different strains of *Lactobacilli* such as *L. acidophilus, L. casei*, *L. plantarum* and *Lactococcus lactis*. Nowadays, microorganisms of different groups or mixed culture are widely used in many probiotic applications as single strains or in combination. However, bacteria belong to *Lactobacilli* and *Bifidobacteria* groups are typical found in many probiotic products for human and animal use (Sanders, 1999). A mixed culture encompasses more than one species. Mixed culture of lactic acid bacteria are currently used in dairy industries for cheese and fermented probiotic milk manufacture. On industrial scale,

mixed culture has not yet being used. In this culture type, the existence of symbiotic relationship among various bacteria has been demonstrated (Moon and Reinbold, 1976). There were lot of research done to produce lactic acid by pure culture and also co-culture (Jawad *et al.,* 2012; Trontel *et al.*, 2011; Farooq *et al.*, 2012). Due to that, this study is focusing more on the used of mixed culture rather than pure culture.

Fermentative production of lactic acid offers the advantages in the utilization of renewable carbohydrates (Patil, *et al.*, 2006). Furthermore, lactic acid bacteria have the property of producing lactic acid from various fermentable carbohydrates. The main problem in producing lactic acid is to recognise the suitable and reliable raw materials. Most of the materials used for lactic acid production are sugar based feedstock such as glucose, molasses, food wastes and corn steep liquor. Jawad *et al.*, (2012) stated that application of agro-industrial wastes in bioprocesses provides an alternative way to replace the refined and costly raw materials. In addition, the bulk use of such materials helps to solve many environmental hazards. However, according to Bulut *et al.,* (2004), the application of microorganisms for the production of lactic acid using cost-effective raw materials is rare. Hence, research efforts are focused on looking for new and effective nutritional sources enabling the achievement of both high substrate conversion and high production.

The production of lactic acid through fermentation technology in industry is mainly dependent on cost of raw material to be used. Therefore, it is mandatory to have a raw material for industrial production of lactic acid with several characteristics such as low cost, minimum level of contaminants, rapid fermentation rate, high lactic acid production yields, little or no by-product formation and year-round availability (Ryu *et al.*, 2003). According to Taskila and Ojamo (2013), chemical purity is mainly depending on the constituents in the fermentation medium especially when cheap materials are being used. The cheap substrate for lactic acid production may come from agricultural wastes containing cellulose and hemicelluloses, which can be converted into soluble sugars by chemical or enzymatic hydrolysis and then by microbial fermentation to lactic acid (Vodnar *et al.*, 2008). Vegetable and fruit processing wastes contain mainly starch, cellulose, soluble sugars and can be used for lactic acid production (Konings *et al.,* 2000). The study focus more on investigating the possibility of producing lactic acid by using banana stem waste instead of glucose, molasses, and other common substrate used. Therefore, consistent to the statement, by using banana stem waste in this study, it proved that production of lactic acid still can be obtained.

In this study, the parameters used for screening are fermentation time, agitation, source of inoculums, temperature and also ratio substrate to inoculums. Most of the screening

procedures generally involve the use of a differential medium occurrence containing a pH indicator (Choudhury *et al.*, 1990). According to the statement, at preliminary experiment, the culture was expected to lower the pH value as they grow and it showed that lactic acid is being produced. A two level factorial design was employed to determine the best condition to produce lactic acid according to the parameter selected. Two-level factorial is widely used in early stages of experiments to screen important factors from a large number of potential factors (Xu *et al.*, 2012). Two-level designs are commonly used to screen factors in the initial stage given a small number of runs.

1.2 Objectives

The following are the objectives of this research :

I. To produce lactic acid by using mixed culture of facultative anaerobic bacteria and banana stem waste.
II. To investigate on the effect of different source of inoculums, temperature, agitation, ratio of substrate to inoculums and fermentation time on the production of lactic acid using mixed culture of facultative anaerobic bacteria and banana stem waste.

1.3 Scope of this research

The following are the scope of this research :

I. Lactic acid fermentation by using banana stems waste and mixed culture of facultative anaerobic bacteria in form of commercially probiotic milk drink.
II. Determination of best condition to produce lactic acid by considering five parameters such as source of inoculums, temperature, agitation, ratio of substrate to inoculums and fermentation time.
III. Determination of glucose concentration in banana stems waste using Dinitrosalicylic Colorimetric (DNS) method and Ultra Violet-Visible (UV-Vis) Spectrometer.
IV. Determination of the lactic acid concentration using High Performance Liquid Chromatography (HPLC).
V. Data analysis of the yield of lactic acid concentration per glucose concentration by Two Level Factorial Design using Design Expert Software.

2 LITERATURE REVIEW

2.1 Overview

The worldwide demand of lactic acid in 2007 was estimated to be 130 000 – 150 000 metric tons per year (John *et al.,* 2007). According to the forecast, the production should increase significantly over the coming years mainly to provide the polylactic acid manufacturing sites and its other applications (Mujtaba *et al.,* 2010). Numerous industries uses lactic acid to produce their desired product such as chemical, food, pharmaceutical, textile, cosmetic and others (Coelho *et al.,* 2010). This was supported by Mudaliyar *et al.,* (2011), who stated that lactic acid is widely used in every segment of food industry. Food, cosmetic, pharmaceutical and chemical industries is proved to be the four major categories for current uses and applications of lactic acid. Worldwide demand on the uses of lactic acid in food related industries almost reached about 85%. In the broad range of application, lactic acid play a vital roles in chemical industries as a precursor for ethyl lactate, propylene oxide, propylene glycol, acrylic acid, 2,3-pentadione and dilactide syntheses (Sheeladevi *et al.,* 2011)

2.2 Mixed Culture of Facultative Anaerobic Lactic Acid Bacteria

Mixed culture has been determined to be effective for certain lactic acid fermentation. Mixed culture of lactic acid bacteria is largely used in dairy industry for manufacturing fermented drinks (Lee, K., 2004). Fermented drinks contain probiotic bacteria that are good to body. National Centre for Complementary and Alternative Medicine (2013) indicated that probiotics are live microorganisms, mostly beneficial bacteria like *Lactobacilli* sp. Probiotics are available to consumers mainly in the form of dietary supplements and foods. In food industries, milk or dietary drink often contains probiotic bacteria such as *Lactobacillus acidophilus* and *Lactobacillus casei*. All the bacteria are gram-positive, facultatively anaerobic, non-motile, non-spore-forming, and rod-shaped lactic acid bacteria. It can be found in dairy and also plant products and in the digestive tract of humans and animals (Gunduz, M., 2005).

Martinez *et al.,* (2013) stated that mixed culture of *Lactobacilli sp.* were employed in lactic acid production to shows better result compared to pure cultures, which is one type of microorganism that usually used in the production of lactic acid. According to the study done

by Abdel-Rahman *et al.,* (2013), amount of lactic acid produced by mixed culture is almost equal with the amount produced by pure culture even though same substrate have been used in the fermentation. Mixed cultures of lactic acid bacteria is more effective than single culturing for improving lactic acid production (Lee, K., 2005).

2.3 Banana Stem Waste

Major factor in the economic production of lactic acid is the raw material cost. From ages, in order to lower the fermentation cost and also producing a pure lactic acid product, pure sugars or edible crops always have been used as substrate (Abdel-Rahman *et al.,* 2013). By products of agriculture industries are one of the alternatives substrate and renewable resources for lactic acid fermentation. According to Litchfield (1998), nowadays, depending on the availability of the substrate in the producing country, variety of carbohydrates is used to produce lactic acid such as starchy and lignocellulosic biomasses.

Fermentative production routes offer advantages of utilization of cheap renewable substrates, low production temperatures and low energy consumption in producing desired products. According to Sheeladevi *et al.,* (2011), various fermentable carbohydrates can act as substrate to produce lactic acid from lactic acid bacteria. Food production utilized traditional feedstock for lactic acid production which is starch based substrates (Bilanović *et al.,* 2011). The profitability of the process could have improved if cheaper substrates such as ligno-cellulosic biomass or agro-industrial wastes are used. Cheap by-products and waste substrates are recommended generally for fermentative production of chemicals to avoid a competition with food industry (Ozalp and Hyman, 2009).

The common used substrates for lactic acid production are glucose, molasses, corn steep liquor and more. Lot of studies have been done using various agriculture resources such as cassava bagasse, apple pomace, date juice, food waste, mango peel and others, Banana stem waste is one of the new renewable carbohydrate sources that have been used as substrate in lactic acid fermentation. As we can see from Table 2.1, Mohapatra *et al.,* (2010) stated that glucose content in the banana pseudo stem is 74.0 %, higher comparing to the other waste. The statement also supported by Sinha *et al.,* (2012), informed that in the pressed juice from banana stem there were 0.41% of carbohydrates per 100 gram. Futhermore, banana stem sap contains 0.191% total sugar, 0.0141% protein and negligible amount of lipids (Feriotti and

Iguti). The carbohydrates existed in the banana stem waste proved that it can act as substrate to produce lactic acid.

Table 2.1 : Chemical composition of different morphologic regions of banana plant

	Pseudostem	**Petioles/mid rib**	**Leaf blade**	**Floral Stalk**	**Leaf Sheaths**	**Rachis**
Glucose	74.0 [a]	68.1 [a]	60.0 [a]	79.8 [a]	74.2 [a]	31.8 [a]
Xylose	13.1 [a]	23.6 [a]	17.5 [a]	9.3 [a]	13.8 [a]	14.0 [a]
Galactose	2.5 [a]	1.1 [a]	3.8 [a]	2.9 [a]	2.2 [a]	1.7 [a]
Arabinose	9.1 [a]	4.9 [a]	15.5 [a]	5.1 [a]	7.5 [a]	4.1 [a]
Mannose	1.3 [a]	1.5 [a]	2.3 [a]	2.2 [a]	1.5 [a]	2.9 [a]
Rhamnose	-	0.8 [a]	0.9 [a]	0.7 [a]	0.8 [a]	0.7 [a]
Lignin	12.0 [a]	18.0 [a]	24.3 [a]	10.7 [a]	13.3 [a]	10.5 [a]
Cellulose	34 - 40 [a]	31.0 [a]	20.4 [a]	15.7 [a]	37.3 [a]	31.0 [a]
Holocellulose	60 - 65 [a]	62.7 [a]	32.1 [a]	20.3 [a]	49.7 [a]	37.9 [a]
Ash	14.0[a]	11.6 [a]	19.4 [a]	26.1 [a]	19.0 [a]	26.8 [a]
Potassium	33.4*	9.4*	11.6 [*]	23.1 [a]	21.4 [*]	28.0*
Calcium	7.5*	32.3*	8.0*	0.6*	5.5 *	0.6*
Magnesium	4.3*	2.9*	1.1*	0.5*	1.9*	0.3*
Silicon	2.7*	7.0*	24.9*	7.8*	2.7*	1.2*
Phosphorous	2.2*	0.7*	0.7*	0.7*	0.9*	1.7*
Pentosans	-	16.2 [a]	12.1 [a]	8.0 [a]	12.3 [a]	8.3 [a]
Starch	-	0.4 [a]	1.1 [a]	26.3 [a]	8.4 [a]	1.4 [a]
Proteins	-	1.6 [a]	8.3 [a]	3.2 [a]	1.9 [a]	2.0 [a]
[a] Expressed in terms of % molar properties; * Expressed in % ash basis						

Table 2.2 : Composition of different parts of banana waste (per 100g)

Waste Product	Moisture (g)	Protein (g)	Fat (g)	Minerals (g)	Fibers (g)	Carbohydrates (g)
Banana peel	79.2	0.83	0.78	2.11	1.72	5.00
Banana stem-central core	95.1	0.30	0.03	1.04	0.68	1.20
Banana stem-outer hard fibrous sheath	91.9	0.12	0.06	0.98	1.81	2.44
Banana stem-pressed juice from stem	98.6	0.05	-	0.63	-	0.41
Sources : Salunkhe and Kadam (1995)						

2.4 Lactic Acid Fermentation

Recently, lactic acid has been produced from a variety of carbohydrates, including starchy and lignocellulosic biomasses, depending on the availability in the producing country (Abdel-Rahman *et al.*, 2013). Sheeladevi *et al.,* (2011) claimed that using various lactic acid bacteria yield different amount of lactic acid under optimized condition of temperature, pH, inoculums level and fermentation period. The statement is supported by Coelho *et al.*, (2010), the temperature and pH are the key of environmental parameters that affect the lactic acid fermentation process. Lactic acid bacteria can grow at temperatures from 5 to 45 °C and, not surprisingly, are tolerant of acidic conditions, with most strains capable of growing at pH 4.4. It is therefore important to determine the temperature and pH at which optimal microbial growth is achieved.

2.5 Factors Used in Lactic Acid Fermentation

In this study, factors that have been effected lactic acid fermentation are pH, fermentation time, temperature and agitation.

2.5.1 pH

The pH has a serious influence on enzyme activities and nutrient assimilations for microorganisms. Increased undissociated lactic acid in accordance with decreasing pH due to lactic acid production is considered to inhibit the fermentation of several lactic acid producers (Abdel-Rahman *et al.,* 2013). According to Busairi (2010), lower pH value indicated that higher lactic acid is being produced. The decrease in pH with time during the fermentation process may be attributed to the production of lactic acid from sugars (Jawad *et al.,* 2013). The fermentation pH is either set at the beginning or then left to decrease due to acid production, and also controlled by base titration, or by extraction, adsorption or electrodialysis of lactic acid (Hofvendahl *et al.,* 2000). Lactic acid production usually have pH varies between 5.0 and 7.0. Kashket (2006) claimed that for *Lactobacillus* strain which known by the abilities to tolerate lower pH have the optimal pH below 5.7. At initial pH 6.5, cell started to utilize glucose earlier and at a faster rate than at other initial pH. Maximum lactic acid concentration was attained at initial pH 6.5 (Boontawan, 2010). The statement is supported by Kohajdová *et al.,* (2005) who stated that a rapid pH reduction in early stages of fermentation is important to obtain a high-quality final product.

2.5.2 Fermentation Time

Fermentation time is one of the critical environmental parameters affecting content, molecular mass, and sugar composition. Utilization of total sugar increased as the fermentation time increased, thus reducing the available sugar content in the media (Farooq *et al.,* 2012). This indicates the utility of sugars during fermentation period. According to Palaniraj *et al.,* (2012), the growth phase is observed from 0 – 55 hours with different initial substrate concentration. Higher initial substrate concentration used causes the growth phase to be slower. Generally, the exponential phase where highest amount of lactic acid produced is between 4 – 40 hours depending on the substrate concentration.

2.5.3 Temperature

Temperature is one of the important factors that affect the growth of microorganism. The characteristics of the microorganism used were affecting the maximum temperature at which the growth rate is the highest. Meanwhile if the optimum temperature was above or over limit the temperature required for certain microbes, they will eventually die or lesser the microbial activity (Tango and Ghaly, 1999). The yield of lactic acid increased with each increase at temperature level of fermentation in between 30 to 40 ^{o}C. The lactic acid production decrease above temperature 45 ^{o}C due to at this temperature the growth not optima therefore the yield become smaller, and the highest yield of lactic acid at 79.8 %, was achieve at 40 ^{o}C. Busairi (2010). The statement is supported by Jawad *et al.,* (2013) where most lactic acid bacteria which are responsible for the conversion of sugar to lactic acid are classified as thermophilic or mesophilic bacteria and usually have an optimum growth between 20 ^{o}C and 40 ^{o}C. Final lactic acid concentration of 5.23 g/L was obtained at 41^{o}C after the fermentation process end indicating that certain amount of sugar is not utilized and hardly converted by the microorganisms (Djukic´-Vukovic *et al.,* 2012). Besides that, Tango and Ghaly (1999) also reported a total of 10 g/L lactic acid was produced at 42^{o}C. Meanwhile, the highest lactic acid production was obtained at 37 ^{o}C and the yield obtained were 28.73 g/L. L. delbrueckii growth seems to grow well at 37 ^{o}C promoting maximum cell concentration and this contributes to maximum lactic acid production (Idris and Suzana, 2006)

Table 2.3 : Previous study on temperature effect in lactic acid fermentation

Temperature (^{o}C)	**Yield of Lactic Acid**		**Reference**
	C (g/L)	Y(g/g)	
20 – 40	Optimum		Jawad *et al.*, (2013)
37	28.73	-	Idris and Suzana (2006)
40	-	0.798	Busairi (2010)
41	5.23	-	Djukic´-Vukovic *et al.*, (2012)
42	10.0		Tango and Ghaly (1999)

2.5.4 Agitation

Different lactic acid bacterial strains differed in their requirement for growth conditions. According to Demirtas *et al.,* (2003), there was 8% increase in growth rate when agitation is increase to 200 rpm to 300 rpm. Meanwhile, some cases where the growth lag was longer due to the constant agitation increase rate, thus taking more time for microbes to adjust under such condition. The increase in agitation speed is expected to result in higher shear stress, causing the fungal to grow in smaller size but increasing the lactic acid production when 0 – 300 rpm is used (Bai, D. M. *et al.,* 2003). This was supported by Tinocco-Valencia *et al.,* (2014), who stated that the shear forces from high agitation can create cell wall rupture, changes in physiological and morphology, biomass concentration, growth rates and also variation in product rate synthesis. However, increase of agitation rates from 50 to 500 rpm, under the experimental condition used, although increase the cell's glucose consumption, did not have significant effect on biomass production, lactic acid concentration and productivity (Gao, T. *et al.,* 2013).

Table 2.4 : Previous study on agitation effect in lactic acid fermentation

Agitation (rpm)	Description	Reference
200 – 500	• Longer lag phase thus affecting microbes growth • Less growth rate increase	Demirtas *et al.,* (2003)
50 - 500	• Increase the cell's glucose consumption • No significant effect on biomass production, lactic acid concentration and productivity	Gao, T. *et al.,* (2013)
0 – 300	• Higher shear stress • Smaller fungal's size • Increase the lactic acid production	Bai, D. M. *et al.,* (2003)

2.6 Previous Study on Lactic Acid Production

Lactic acid production using *Kluyveromyces marxianus* (IFO 288), *Lactobacillus delbrueckii ssp. bulgaricus* (ATCC 11842) and *Lactobacillus helveticus* (ATCC 15009) individually or as mixed culture on cheese whey in stirred or static fermentation conditions was evaluated. The highest lactic acid concentrations were achieved when *K. marxianus* yeast was combined with *L. delbrueckii ssp. bulgaricus* (Plessas *et al.*, 2007). Lee (2005) observed that mixed cultures of lactic acid bacteria maybe more effective than single culturing for improving lactic acid production.

Oh *et al.,* (2005) has been studied on the comparison in lactic acid production by using three different substrates which are wheat, corn and barley. All the research took place about 48 hours with temperature of 38^{o}C and 200 rpm speed. The highest production (0.94 g/g) was obtained when barley and corn is used as substrate. However, the tiny margin between all lactic acid yields by using the three substrates showed that the content of carbohydrates in this case was glucose, is relatively have almost the same amount. The utilization of the sugar content is parallel throughout the fermentation process, thus producing different amount of lactic acid.

Meanwhile, Farooq *et al.,* (2012) reported that the concentration of lactic acid produced with single culture *Lactobacillus delbrueckii*, was 77.6 g/L. Significantly the highest lactic acid production took place after 7 days of fermentation with 42^{o}C temperature and no agitation used. During second and third days of fermentation, rapid increase in production of lactic acid was observed until day 7, meanwhile at the 8th, the yield started to show a non significant decrease, correspond to the utilization of sugar content in the sugarcane molasses.

Mixed cultures or co-cultures of lactic acid producing microorganisms may increase the conversion efficiency of substrate (Cui *et al.*, 2010). Lee (2005) stated that the biotechnological process, by a mixed cell culture, has the advantage of better growth of cells, higher lactic acid production and lower nutrient consumption.

On the other hand, Nancib *et al.,* (2009) investigated the production of lactic acid from date juice by the single and mixed culture system of *Lactobacillus casei* and *Lactococcus lactis*. Using the same parameters for both single and mixed culture with only 19 hours fermentation time, 150 rpm agitation and operating at 30 ^{o}C, there were distant differences in lactic acid production. The concentration of residual glucose and fructose for

single culture is higher compared to mixed cultures which are 17.8; 5.6 and 4.0; 0.0 respectively. Lactic acid concentration of 60.3 g/L and glucose efficiency of 96% were achieved with the mixed culture whereas 53 g/L of lactic acid concentration and 82.2% of glucose efficiency were obtained using single culture. The statement also similar with Taniguchi *et al.*, (2004), where a co-culture of *Entrococcus casseliflavus* and *L.casei* was reported to produce 95 g/L of lactic acid after fermentation completed.

Jawad *et al.*, (2013) evaluated the bio-fermentation process by producing lactic acid from mango peels. Natural mixed lactic acid bacteria act as microbes in the breakdown of peel polysaccharides to glucose and the eventual conversion of the glucose into lactic acid by microorganisms in the fermentation broth throughout the process. The operational variables were carefully observed and showed that the production of lactic acid depended significantly on those factors, which are initial pH, temperature and incubation time. The results for maximum production of lactic acid (17.4 g/L) achieved at initial medium pH of 10; incubation time of 6 days; and at a temperature of 35^{o}C.

Table 2.3 summarize all different substrate for lactic acid fermentation using pure and mixed culture. According to Garde *et al.*, (2002), by using mixed culture, the lactic acid yield is 0.95 g/g, almost similar with the result from using pure culture.

Table 2.5 : Different Substrate for Lactic Acid Fermentation Using Pure and Mixed Culture

Fermentation Substrates	Cultures	Glucose Efficiency	Lactic Acid Produced	Reference
Barley	Pure	65.0 ± 1.7 g/g	0.94 ± 0.02 g/g	Oh *et al.*, (2005)
Wheat	Pure	52.5 ± 1.4 g/g	0.93 ± 0.01 g/g	
Corn	Pure	67.5 ± 1.5 g/g	0.94 ± 0.01 g/g	
Sugarcane Molasses	Pure	-	77.6 g/L	Farooq *et al.*, (2012)
Dates Juice	Pure	82.2 g/L	53.0 g/L	Nancib *et al.*, (2009)
	Mixed	96.0 g/L	60.3 g/L	
Mango Peel	Mixed	17.4 g/L	-	Jawad *et al.*, (2013)
Cheese Whey	Mixed	-	0.35 g/g	Plessas *et al.*, (2008)
Wheat Straw	Mixed	-	0.95 g/g	Garde *et al.*, (2002)
Mango Peel	Mixed	-	17.4 g/L	Jawad *et al.*, (2013)

2.7 Screening

2.7.1 Fractional Factorial Design (FFD)

According to Kiew, P. L. *et al.,* (2013), two level fractional factorial design is a popular experimental design and mostly applied in engineering analysis. FFD allows possible consideration of multitudinous factors and determine the most relevant factors from all the outcomes. The statement is supported by Khalil, M. *et al.,* (2011) where FFD is said to investigate the effect of tested independents variables to the response within the investigation range. FFD also is a technique where not only the determination of the influence of several variables on the response but also estimating the overall main factor effects and interaction of different factors (Golshani, T. *et al.,* 2013). Jawad *et al.,* (2013) stated that the factorial design was employed to study the effect of independent variables and the level of selected factors can be chosen based on preliminary experiments.

In this study, based on preliminary experiments, five different factors was tested which are temperature, agitation, source of inoculums, ratio of substrate to

inoculums and fermentation time.. The levels of selected factors were stated according to preliminary experiments as below :

Table 2.6 : Process variables and levels for FFD

Process Variables	**Levels**		
Temperature (^{o}C)	30	35	40
Fermentation time	20 hour	30 hour	40 hour
Agitation (rpm)	No	Yes	
Sources of inoculums	Yakult	Nutrigen	
Ratio of Substrate to Culture	4:1	2 : 3	

The experiment design protocol was contrived with the aid of the software Design Expert.

3 METHODOLOGY

3.1 Overview

This chapter discussed the materials and methods adopted in the experimental work. This chapter explained the fermentation process and also the analysis of lactic acid produced. The subchapter covers in this chapter was substrate preparation, inoculums preparation, preliminary study, experimental design, analysis, summary of experimental design and also validation experiment. These methodologies were being used thoroughly in this study.

A schematic structure of the whole process flow has been constructed and is illustrated in Figure 3.1. As the starting point, the banana stem was collected and prepared for fermentation process following by DNS Method. Then, fulfilling the first objective, inoculums was prepared in term of probiotic milk drink. Screening factors were decided beforehand and using Design Expert 6.0 software, the experimental runs was done by full factorial design (FFD). The effect of all five factors, agitation, source of inoculums, ratio of substrate to inoculums, fermentation time and temperature on lactic acid fermentation were investigated to achieved the second objective. After fermentation process, the samples were collected and ready to be analyzed by High Performance Liquid Chromatography (HPLC). Then, using the software again, the R^2 value was calculated to see whether the data collection was valid or not. The last step would be the experimental validation run. It is to confirm and to find the best condition to produce lactic acid from banana stem waste.

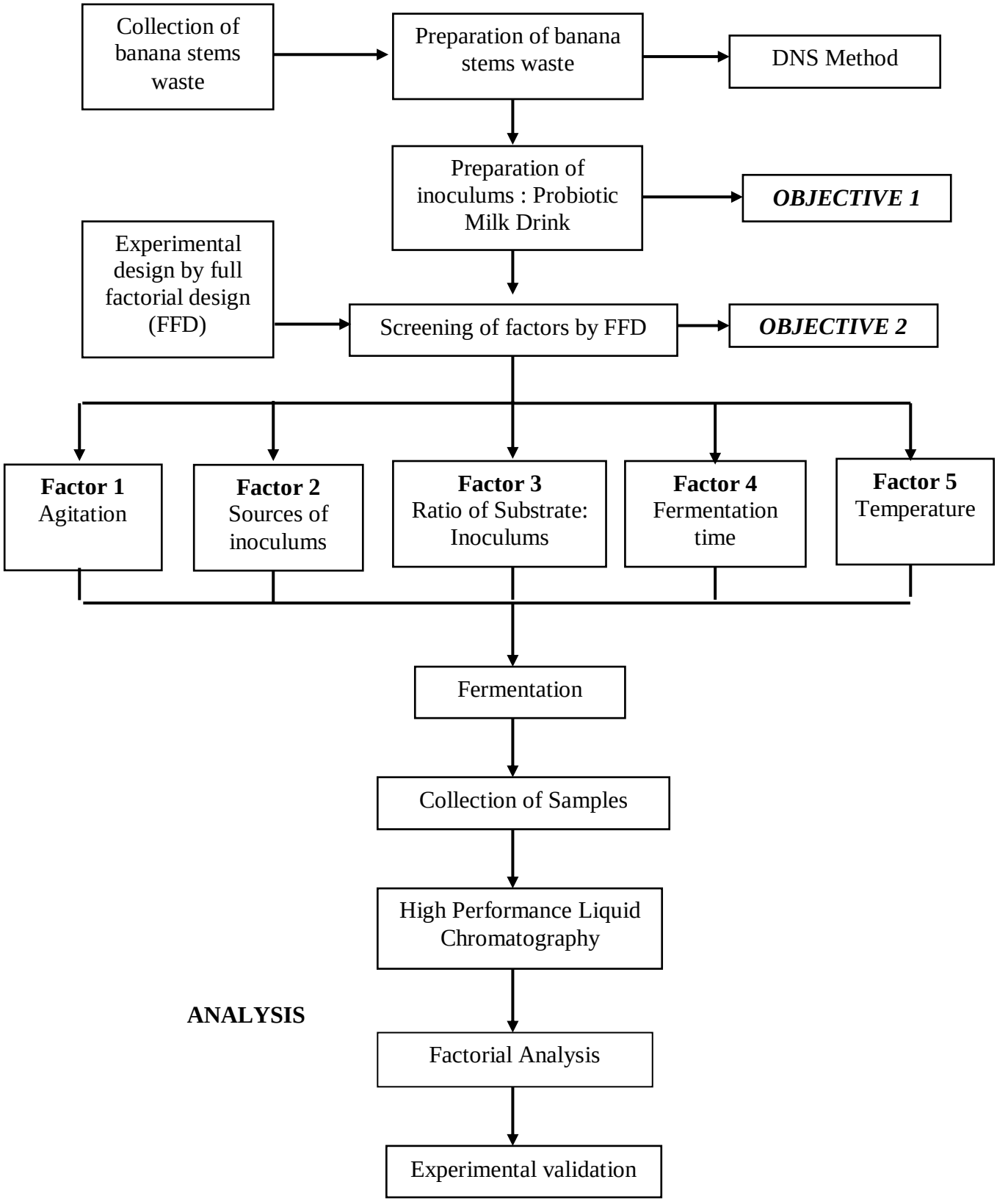

Figure 3.1 : Flow chart process of experiment